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Comparison of trichloroethylene reductive dehalogenation by microbial communities stimulated on silicon-based organic compounds as slow-release anaerobic substrates

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Abstract

Microcosm studies were conducted to demonstrate the effectiveness of tetrabutoxysilane (TBOS) as a slow-release anaerobic substrate to promote reductive dehalogenation of trichloroethylene (TCE). The abiotic hydrolysis of TBOS and tetrakis(2-ethylbutoxy)silane (TKEBS), and the biotic transformations of the hydrolysis products from both were also investigated. Comparison of TCE reductive dehalogenation was performed with microbial communities stimulated from three different sites: Site 300 Lawrence Livermore National Laboratory (LLNL), CA, Point Mugu Naval Weapons Facility, CA, and the Evanite site in Corvallis, OR. Poisoned microcosms showed that 1 mol of TBOS slowly and abiotically hydrolyzes to 4 mol of 1-butanol, while the live microcosms showed the 1-butanol ferments to butyrate and/or acetate, producing H_2. The hydrolysis of TBOS and TKEBS was abiotic and not enhanced by biotic processes under the anaerobic conditions of these tests. Hydrogen consumption was correlated with reductive dehalogenation, indicating it served as an electron donor for reductive dehalogenation. TBOS was found to be a slow-release anaerobic substrate to support long-term dechlorination of TCE to ethylene in Point Mugu microcosms, and in the LLNL microcosm bioaugmented with the Evanite culture. Electron mass balances showed most of the electron flow went into the creation of organic acids, especially acetate, and the production of methane. Electron efficiencies for reductive dechlorination were as high as 14% based on the electrons used for dechlorination to the total electrons associated with the mass of TBOS and TKEBS hydrolyzed. Rates of TBOS hydrolysis increased with greater TBOS concentrations as a light nonaqueous-phase liquids (LNAPL). These results indicate that TBOS has promise as an effective anaerobic substrate for remediating a wide range of CAH concentrations at different CAH contaminated sites. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Reductive dehalogenation; Trichloroethylene; Tetrabutoxysilane; Hydrolysis

1. Introduction

Trichloroethylene (TCE) is one of the most common groundwater contaminants in the United States [1]. Under anaerobic conditions, TCE can be reductively dechlorinated to *cis*-1,2-dichloroethylene (*c*-DCE), vinyl chloride (VC), and finally ethylene or ethane [2,3]. However, the extent of dechlorination differs among contaminated sites. Anaerobic dechlorination has been drawing attention for the in situ bioremediation of PCE and TCE [4,5]. Furthermore, anaerobic dechlorination has potential for remediating high concentrations of TCE associated with dense nonaqueous-phase liquids (DNAPLs) contamination [6] and for enhancement of DNAPL dissolution [7,8].

Many organic compounds, such as organic acids and alcohols, have been studied as potential electron donors to promote anaerobic dechlorination [9]. Complex

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substrates are fermented to less complex substrates, producing H_2, which can serve directly as an electron donor for reductive dechlorination [10]. However, other microorganisms including methanogens, homoacetogens, and sulfidogens compete for H_2 as an electron donor in the subsurface environment [3]. Enhanced bioremediation may require the use of substrates that are efficiently used for anaerobic dechlorination [5]. Many researchers have physiologically and thermodynamically studied hydrogen competition for a variety of hydrogenotrophic microbial consortiums. Dechlorinators were shown to have a lower hydrogen threshold concentration of 0.3 nM compared to acetogens (336–3640 nM), methanogens (5–95 nM), and sulfidogens (1–15 nM) [11]. Other studies reported a hydrogen threshold in the range of 0.05–11 nM for dechlorinators [4,5], suggesting that anaerobic dechlorinators can outcompete the other hydrogenotrophs at low hydrogen concentrations. In addition to the physiological hydrogen threshold, free energy calculations indicate a lower hydrogen threshold for dechlorinators than for other hydrogenotrophs [4]. Several studies revealed that an electron donor that is slowly and steadily transformed to maintain low H_2 concentrations could optimize anaerobic dechlorination and limit methanogenesis [4,5,9,12,13].

Tetrabutoxysilane (TBOS [$Si(CH_3CH_2CH_2CH_2O)_4$]) and tetrakis(2-ethylbutoxy)silane (TKEBS [$Si(CH_3CH_2CH(CH_2CH_3)CH_2O)_4$]) are silicon-based compounds having four oxygen branches with butyl and 2-ethylbutyl groups, respectively. These compounds were observed to act as LNAPLs and mix well with CAHs (Table 1) [14]. They have a low solubility in water and thus slowly dissolve. The silicon-based compounds have been shown to support anaerobic dechlorination of TCE at chlorinated solvent-contaminated sites [15,16] and with laboratory cultures [17]. Evidence for the transformation pathway of alkoxysilanes, mainly TKEBS, and their potential to serve as substrates for promoting TCE transformation was discovered through analysis of groundwater from Site 300 at Lawrence Livermore National Laboratory, CA (LLNL) [18]. Based on the field-monitoring data, TBOS and TKEBS were observed to slowly hydrolyze to 1-butanol and 2-ethylbutanol, respectively, and ferment to butyrate and 2-ethylbutyrate [15,16,18]. However, the abiotic hydrolysis of TBOS and the biotic fermentation of the hydrolysis products needed additional study, as well as the effect of TBOS addition on reductive dehalogenation. The effectiveness of TBOS addition as a slow-release substrate with microbial communities from different sites was also needed to be evaluated. During the fermentation of the alcohols and acids, H_2 is produced and becomes a potential electron donor for the dechlorinators [3,11]. Butyrate, one potential fermentation product, has been shown to be an excellent slow-fermenting substrate among other fatty acids to produce hydrogen needed for dehalogenation reactions [9].

Table 1
Physical properties of tetraalkoxysilanes (adapted from [18])

Property	Compound	
	TBOS ($C_{16}H_{36}O_4Si$)	TKEBS ($C_{24}H_{52}O_4Si$)
Molecular weight (g/mol)	320.5	432.8
Boiling point (°C)	115	166
Density (g/ml)	0.899	0.892
Refractive index	1.41	1.43
Solubility (mg/l at 20°C)	<1	<1
Viscosity (cSt[a] at 38°C)	2.33	4.35
Surface tension (dyn/cm)	22.8	22.8

[a] cSt: centistoke = 0.01 cm^2/s.

The objectives of this study were: to investigate the effectiveness of TBOS as a slowly hydrolyzing substrate to drive the enhanced anaerobic transformation of TCE; to evaluate the abiotic hydrolysis of TBOS; and to determine the biotic transformation of the hydrolysis products from TBOS. This study focused on TBOS addition as a slow-release substrate, since it hydrolyzes more rapidly than TKEBS. Butyrate, as a potential fermentation product of TBOS, has been studied in greater detail than ethylbutyrate produced from TKEBS. Some TKEBS was present in groundwater from Site 300 LLNL, so its fate was also monitored.

Comparison of TCE reductive dehalogenation was performed with microbial communities stimulated from three different sites; Site 300 LLNL, CA, Point Mugu Naval Weapons Facility, CA, and the Evanite site in Corvallis, OR, and stimulated with the silicon-based organic compounds. The microbial community stimulated from LLNL Site 300 groundwater showed incomplete dechlorination of TCE to *c*-DCE without methanogenesis. The communities stimulated from the two other sites had the ability to dechlorinate TCE to ethylene. In the Point Mugu microcosms, a very slow-dechlorination rate from VC to ethylene was observed.

2. Materials and methods

2.1. Chemicals

TKEBS (United Chemical Technologies Inc., 97%, Bristol, PA) and TBOS (Aldrich Chemical, 97%, Milwaukee, WI) were used as substrates and in the preparation of analytical standards. Dichloromethane (DCM) (Fisher Scientific Co., 99.9% HPLC Grade, Pittsburgh, PA) was used for solvent extraction of

TBOS, TKEBS, and alcohols prior to gas chromatographic (GC) analysis. 2-ethylbutanol (Aldrich Chemical, 98%, Milwaukee, WI), 1-butanol (Aldrich Chemical, 99.8%, HPLC grade, Milwaukee, WI), 2-ethylbutyric acid (Aldrich Chemical, 99%, Milwaukee, WI), sodium butyrate (Aldrich Chemical, 98%, Milwaukee, WI), and sodium acetate (Aldrich Chemical, 99+%, Milwaukee, WI) were used for preparing analytical standards. Sodium carbonate (99%) was obtained from Mallinckrodt Co. (Paris, KY) for preparing buffer solution (pH 7.2) in abiotic hydrolysis experiments.

2.2. Analytical methods

TKEBS, TBOS, 2-ethylbutanol, and 1-butanol were determined by GC analysis after solvent extraction. An aqueous sample (0.5 ml) was taken after actively shaking the microcosms. The aqueous samples were extracted with 1 ml of DCM with vigorously mixing for 10 min on a vortex mixer and centrifuged at 10,000 rpm for 5 min. The extract (1 μl) was introduced into a HP-5890 GC equipped with a flame ionization detector (FID) and Rtx-5 column (30 m × 0.32 mm, 0.25-μm film) from Restek, Inc. (Bellefonte, PA). The oven temperature program was set as follows: 35°C for 5 min; increased at 40°C/min to 300°C; kept at 300°C for 4 min. Helium served as the carrier gas (30 ml/min) for the column.

TCE, *c*-DCE, vinyl chloride, ethylene, and methane were measured by GC analysis via separation on a 30 m × 0.53 mm GS-Q column (J&W Scientific, Folsom, CA). A microcosm headspace sample (20–100 μl) was injected into an HP-6890 GC equipped with a photoionization detector (PID) and FID connected in series. The GC oven was initially set at 40°C for 2 min, heated at 25°C/min to 160°C and 15°C/min to 220°C, and kept at 220°C for 1 min. Hydrogen and carbon dioxide concentrations in microcosm headspace gas samples (200 μl) were determined using an HP-5890 GC with a thermal conductivity detector (TCD), operated isothermally at 220°C. The headspace gas samples were chromatographically separated with a Carboxen 1000 column (15 ft × 1/8 in, Supelco, Bellefonte, PA). Argon gas was used as a carrier gas at 15 ml/min for operation of HP GC. The hydrogen detection limit was 4 nM (liquid concentration basis) with the TCD measurement. Volatile acids in aqueous samples were determined using high performance liquid chromatography (HPLC). A Dionex DX 500 HPLC (Dionex, Sunnyvale, CA) equipped with an UV absorbance detector operated at 210 nm. Separation of acids was made using a Phenomenex Rezex ROA-Organic Acid column (300 × 7.8 mm) and 0.013 N H_2SO_4 (pH 3.0) as an eluent at the flow rate of 0.5 ml/min.

A six-point linear standard calibration curve was constructed with triplicate samples prior to initial sampling for all chromatographic analyses. For each sampling event, one point check was carried to determine the stability of the detectors and for recalibration purposes.

2.3. Culture enrichment and growth

The culture used for bioaugmentation was enriched from groundwater from the Evanite site in Corvallis, OR. The enrichment culture was grown in a batch reactor bottle on butanol in groundwater (no addition of nutrients or salts) at 30°C with continuous shaking at 200 rpm over the 6 month incubation period, with repeated additions of TCE. After each addition of TCE was converted completely to ethylene, the batch bottle was purged with the mixed gas of N_2 (90%) and CO_2 (10%) and neat TCE of 6–8 mg/l was added to achieve an aqueous concentration. Neat butanol was also added to maintain aqueous concentration of 100–300 mg/l. A fairly constant TCE dechlorination rate was obtained after 150 days of incubation. Additional tests showed the enrichment culture was capable of completely transforming TCE to ethylene with active methanogenesis with lactate, propionate, butyrate, and butanol added as electron donors.

2.4. Microcosm preparation

Batch microcosm bottles were constructed with serum bottles (Wheaton Industries, Millville, NJ) fitted with rubber-lined caps and butyl rubber septa (Wheaton Industries, Millville, NJ) (Table 2). The microcosms were constructed in an anaerobic glove box filled with 10% H_2 and 90% N_2. The site and microcosm volumes were as follows: LLNL Site 300 (316 ml), Pt. Mugu (1060 ml), and the LLNL bioaugmentation study (155 ml). LLNL microcosms consisted of 250 ml of groundwater (well D3) and 66 ml of headspace. No solids were added due to the unavailability of core material. The bioaugmentation microcosms contained 70 ml of LLNL groundwater, 5 ml of an enriched liquid culture from the Evanite site, and 80 ml of headspace. Pt. Mugu microcosms contained 750 ml of groundwater, 200 ml of aquifer solids, and 110 ml of headspace. After construction in the glove box, the microcosm headspace hydrogen was removed by purging with the mixed gas of N_2 (90%) and CO_2 (10%) that was treated in a tube furnace to remove trace oxygen. TBOS and TCE were added to each microcosm bottle as neat liquid or diluted aqueous solution (Table 2). TBOS was added at a high concentration to the Pt. Mugu microcosm since high sulfate concentrations (~1000 mg/l) were initially present in the groundwater. TKEBS was detected in the ambient groundwater used to construct LLNL microcosms, but was not present in groundwater later used to construct LLNL microcosms bioaugmented with the

Table 2
Initial experimental conditions of microcosm bottles

	LLNL (at day 0)	LLNL with the Evanite culture (at day 0)	Pt. Mugu (at day 0)
TCE, μM (mg/l)	381 (50)[a]	15 (2)[b]	91 (12)[a]
TBOS, μM (mg/l)	156 (50)[a]	281 (90)[a]	3121 (1000)[a]
Methanogenesis	No	Yes[c]	Yes
Groundwater (ml)	266	75[d]	750
Headspace (ml)	66	80	110
Aquifer solids (ml)	—	—	200

[a] Neat liquid added.
[b] Stock solution added.
[c] Methanogenesis from bioaugmentation of the Evanite culture.
[d] 70 ml of LLNL groundwater + 5 ml of the Evanite enrichment culture.

Evanite culture. Duplicate batch microcosms were incubated for each experiment. Control microcosms were constructed in the same manner as the live microcosms, but were poisoned with 25 mg/l mercuric chloride ($HgCl_2$). All microcosms were incubated with continuous shaking at 200 rpm in a 30°C environmental chamber.

For abiotic hydrolysis experiments, 0.5 g of Ottawa sand (sieved between no. 30 and 50) was placed in 2 ml glass vials, and 0.2 ml carbonate buffer solution (pH 7.2) was added with different TBOS concentrations. These glass vials were capped with Teflon-coated butyl rubber septa (Wheaton Industries, Millville, NJ) sealed in place with an aluminum crimp cap. A total of 24 bottles were prepared for each concentration, and incubated at 20°C for 80 days with continuous shaking at 200 rpm. Three vials from each concentration were sacrificed for every analysis of TBOS and 1-butanol. The abiotic hydrolysis rates of TBOS were obtained by a simple linear regression fit of 8 data points (each data point was the average of the triplicates).

3. Results and discussion

3.1. Enhanced TCE reduction driven by TBOS in LLNL microcosms

The poisoned microcosms were used to track the abiotic hydrolysis of TBOS and TKEBS. The background site groundwater initially had several fermentation products present. The hydrolysis of TBOS and the accumulation of its product, 1-butanol, are shown in Fig. 1(a). 2-ethylbutanol was also produced from the slow hydrolysis of TKEBS present in the site groundwater. The abiotic hydrolysis rate of TBOS is about 10 times faster than that of TKEBS, consistent with previous laboratory studies [18]. Over 100 days of incubation, 35–40 μmol (11.2–12.8 mg) of TBOS disappeared and 140–160 μmol (10.4–11.8 mg) of 1-butanol was produced, while 4–5 μmol (1.7–2.2 mg) TKEBS disappeared, resulting in the production of 15–16 μmol (1.5–1.6 mg) of 2-ethylbutanol. These results are consistent with a stoichiometric ratio of 4 mol of alcohol formed for each mole of alkoxysilanes hydrolyzed. The acids in the background site groundwater (acetate, butyrate, and 2-ethylbutyrate) and hydrogen were constant over the 180-day incubation period of the poisoned control. The stepwise additions of TCE at 0, 133, and 160 days show no dechlorination to *c*-DCE (Fig. 1(c)), indicating $HgCl_2$ poisoning inhibited the biological fermentation reactions and the dechlorination process. The duplicate control showed near identical results.

Fig. 2 shows the fermentation of the alcohols and organic acids produced from the hydrolysis of TBOS and TKEBS, resulting in H_2 production in a biologically active LLNL microcosm. Dechlorination of TCE to *c*-DCE was correlated with hydrogen consumption. TCE was transformed to *c*-DCE within 25 days of incubation, and hydrogen began to accumulate after TCE was transformed to *c*-DCE. 1-butanol, butyrate, and acetate concentrations started to accumulate and increased until the second addition of TCE at 133 days, while little change in 2-ethylbutanol concentration was observed. Aqueous hydrogen concentrations (600–1500 nM from 44 to 134 day, total H_2 masses of 2–6 μmol, respectively) were maintained at levels consistent with H_2 threshold concentrations for acetogenesis [11]. After the second addition of TCE, the concentrations of butyrate and H_2 decreased, and acetate increased. Decreases in hydrogen concentration occurred during the period when TCE was transformed to *c*-DCE, indicating hydrogen was used as an electron donor to support TCE dechlorination. Aqueous hydrogen concentration levels decreased to below the detection limit of 4 nM, consistent with dehalogenation reactions. Hydrogen concentrations increased after TCE was transformed to *c*-DCE as a result

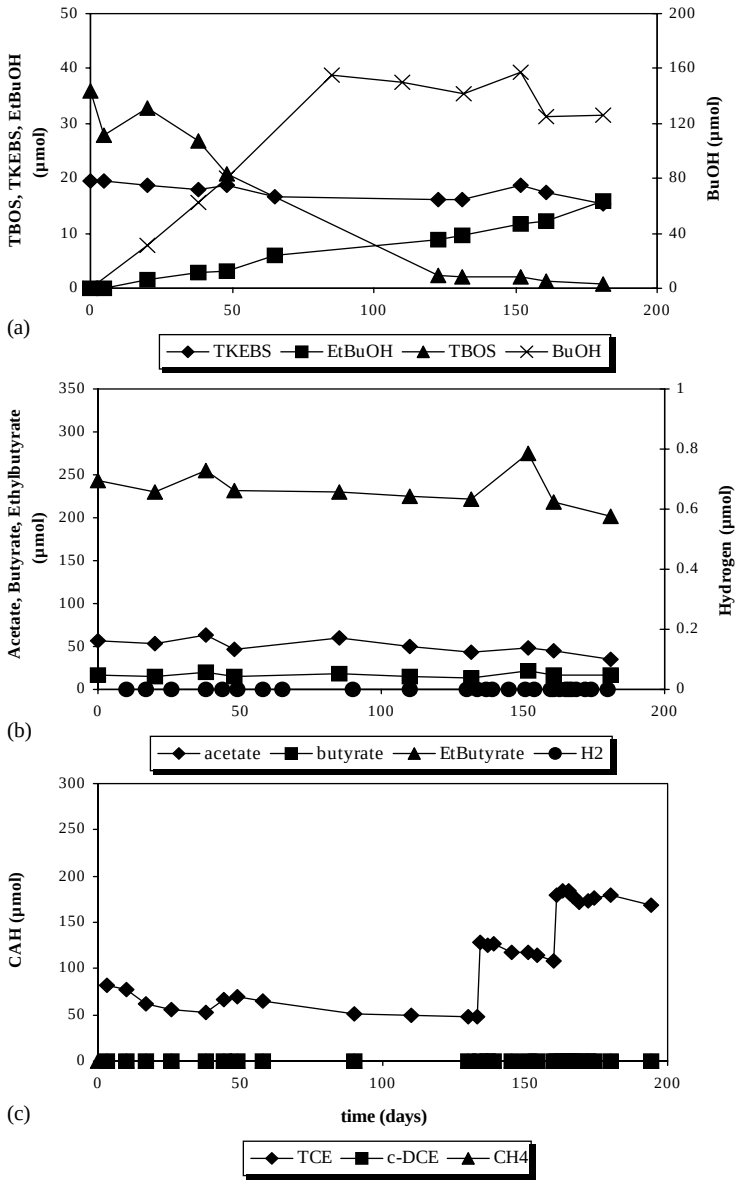

Fig. 1. Poison control microcosm containing groundwater from Site 300 of LLNL: (a) TBOS, TKEBS, and their hydrolysis products, (b) acids from fermentation process and hydrogen, and (c) CAHs. The initial TCE mass of 80 µmol is equal to an aqueous concentration of 290 µM. The hydrogen detection limit of 4 nM represents a total mass of 0.015 µmol.

of butanol and butyrate fermentation. The third addition of TCE showed similar results as the second, with respect to hydrogen consumption and accumulation. The rates of TCE dechlorination at 133 and 160 days were much faster than the initial transformation at 20 days, but the transformation did not proceed past *c*-DCE, which is consistent with the field-monitoring results. No production of methane was observed during the course of the experiment.

3.2. Bioaugmentation microcosm study with TBOS addition

TBOS was also evaluated as an anaerobic substrate in a LLNL microcosm that was bioaugmented with the Evanite culture that can completely dechlorinate TCE to ethylene. The slow hydrolysis of TBOS in the bioaugmented microcosm is shown in Fig. 3(a). TKEBS was not present in the site groundwater used to construct

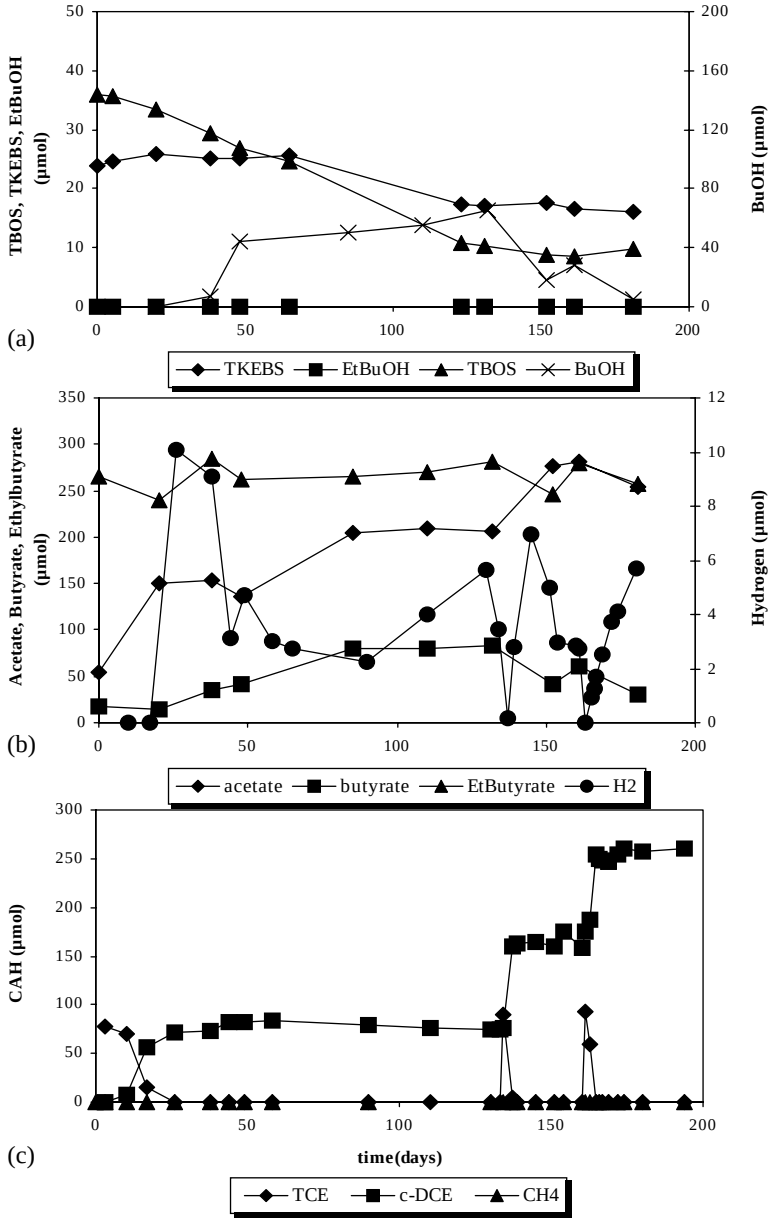

Fig. 2. Live microcosm amended with TBOS to groundwater from Site 300 of LLNL: (a) TBOS, TKEBS, and their hydrolysis products, (b) fermentation products and hydrogen, and (c) CAHs. The initial TCE mass of 80 µmol is equal to an aqueous concentration of 290 µM. The hydrogen detection limit of 4 nM represents a total mass of 0.015 µmol.

these microcosms. Butanol was not detected, but butyrate and acetate were observed as fermentation products of butanol. In the beginning of the experiment, butyrate increased along with an increase in H_2 concentration. When *c*-DCE was transformed to VC, butyrate and H_2 rapidly decreased and were maintained at decreased levels during the slow transformation of VC to ethylene. The concentration of acetate increased throughout most of the study. The decrease in acetate concentration observed after 150 days coincided with accelerated methanogenesis and was likely associated with acetotrophic methanogenesis. The bioaugmentation resulted in methanogenesis that was absent in the LLNL microcosms (Fig. 2c).

As shown in Fig. 2(c), the groundwater microcosm from LLNL shows limited dechlorination of TCE to *c*-DCE

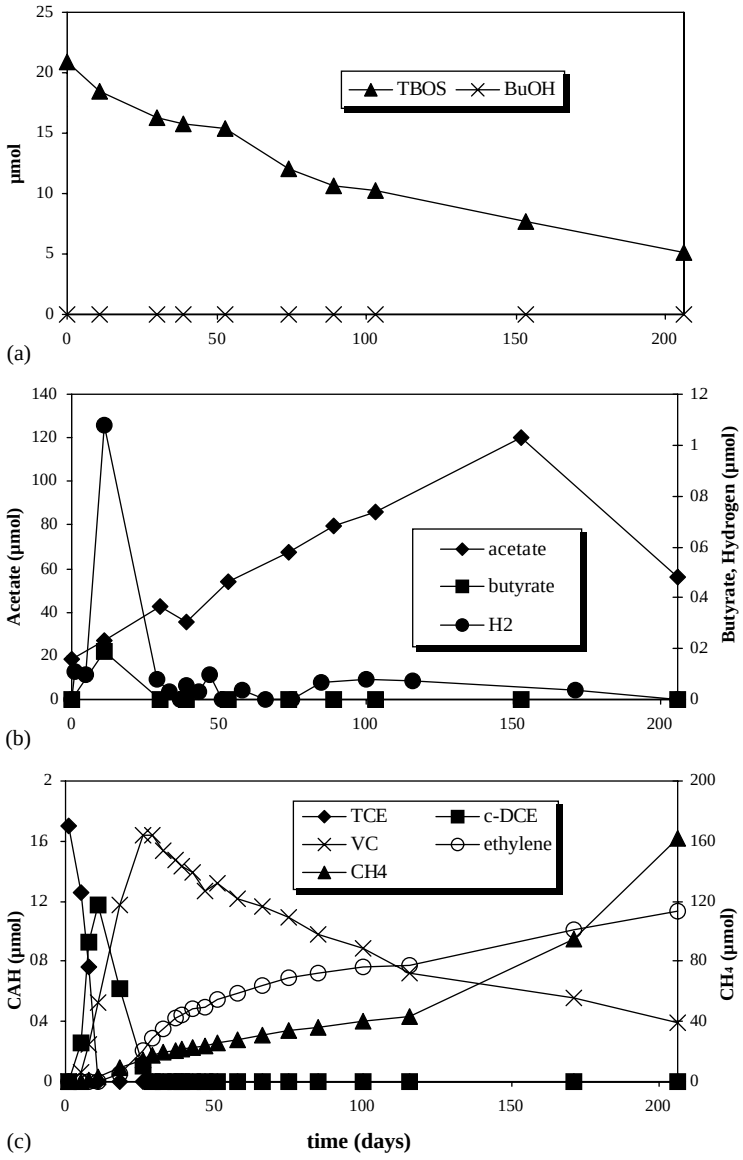

Fig. 3. Groundwater microcosm from the LLNL Site 300 bioaugmented with the enriched mixed culture from the Evanite site. The initial TCE mass of 1.7 µmol is equal to an aqueous concentration of 17.2 µM in the microcosm. The hydrogen detection limit of 4 nM represents a total mass of 0.017 µmol.

over a prolonged incubation period. However, when LLNL groundwater was bioaugmented with the Evanite culture grown on butanol, dechlorination to ethylene was observed as a result of a single addition of TBOS (Fig. 3(c)). *c*-DCE was rapidly dechlorinated to VC, and VC was slowly dechlorinated to ethylene. Ethylene continuously accumulated in the microcosm bottle even though the production rate was very slow compared to the dechlorination of TCE and *c*-DCE. Aqueous hydrogen concentrations were also maintained at levels of 8.5 nM (a total mass of 0.04 µmol; average value between 29 and 116 days) in the bioaugmented microcosm.

3.3. Pt. Mugu microcosms amended with TBOS as a sole anaerobic substrate

The Pt. Mugu microcosms were initially amended with TBOS of 2341 µmol (1000 mg/l = 3121 µM) as a sole substrate. Higher TBOS concentrations were used than in the LLNL microcosms, since sulfate (~1000 mg/l)

was present in the groundwater. The microcosms were operated over a period of 900 days, generating effective anaerobic conditions with only the initial addition of TBOS.

Fig. 4 shows the dechlorination of TCE to VC and a slow rate of ethylene formation. Dechlorination was delayed for about 60–70 days while the background sulfate (~1000 mg/l) and nitrate (trace amounts) were reduced to low concentration levels (data not shown). One period of incubation (505–575 days) was chosen to show how TBOS supports the reductive dechlorination of TCE to ethylene (Fig. 4). At 505 days, the microcosm headspace was purged and TCE was added. The abiotic hydrolysis of TBOS, the production of acetate and butyrate from butanol, and the maintenance of hydrogen concentrations are shown. The accumulation of butanol was not observed over the period, indicating it was rapidly fermented to butyrate and potentially

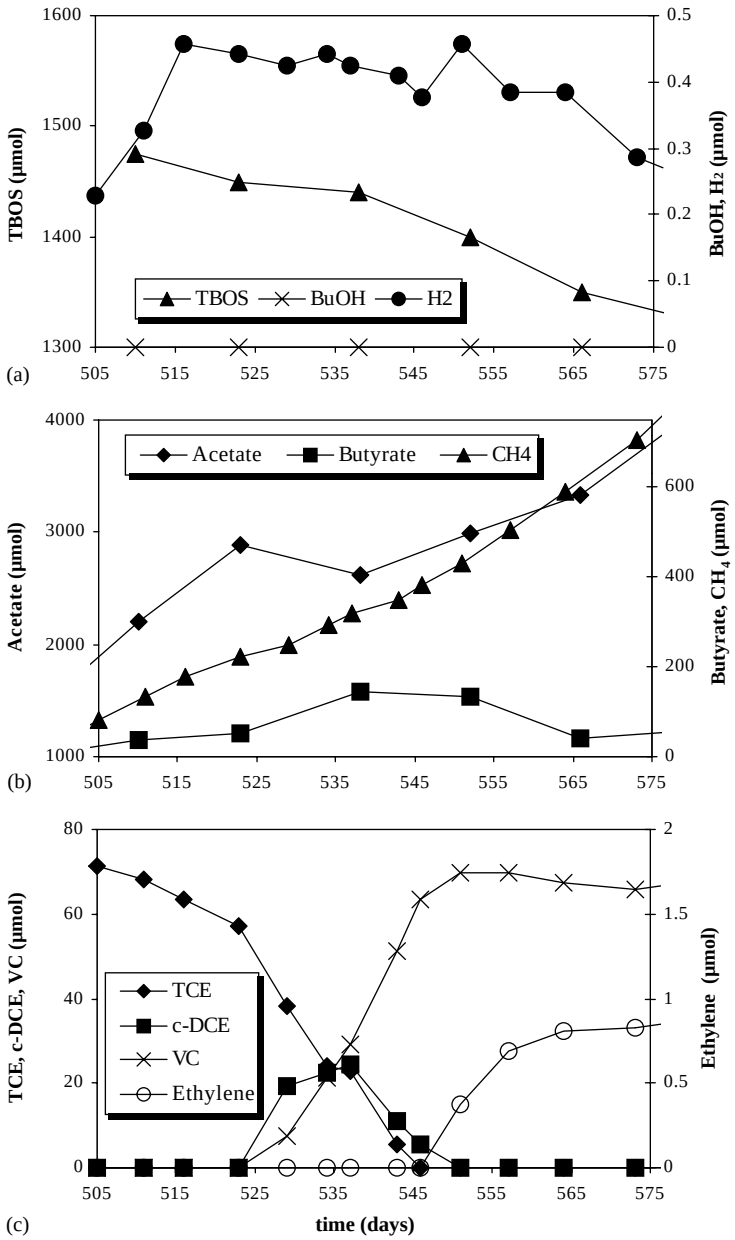

Fig. 4. Pt. Mugu microcosm bottle amended with TBOS as an anaerobic substrate. The initial TCE mass of 71 μmol is equal to an aqueous concentration of 89 μM. The hydrogen detection limit of 4 nM represents a total mass of 0.025 μmol.

acetate. Aqueous hydrogen concentrations remained relatively constant (36–72 nM) as TCE was actively transformed. The fermentation of butanol and butyrate likely maintained the constant and high hydrogen concentrations. Acetate concentration continuously increased over the time course of the experiment, while butyrate increased from 525 to 535 days and then started to decrease. VC was dechlorinated to ethylene at a very slow rate. Previous microcosm studies for Pt. Mugu have also shown slow transformation of VC to ethylene [19].

3.4. TBOS transformation pathway

Based on the results of this study, the pathway for the abiotic and biotic transformation of TBOS is presented in Fig. 5. TBOS abiotically hydrolyzes to 1-butanol, which ferments to butyrate and/or acetate producing H_2 during the fermentation process. One mole of TBOS was shown to produce 4 mol of 1-butanol through abiotic hydrolysis, suggesting that butanol is the main hydrolysis product. Butanol ferments to butyrate and/or acetate, consistent with the observations of Eichler and Schink [20]. Rapid fermentation of butanol to butyrate was observed, since butanol did not accumulate in the microcosms. Butyrate could then serve as a slowly fermenting substrate to stimulate reductive dechlorination. Butyrate has been reported as a low-H_2-producing substrate that favors dechlorination rather than methanogenesis [9]. However, the H_2-forming fermentation of butanol to butyrate will produce higher H_2 levels as shown in the standard free energy change ($\Delta G^\circ = 16.5$ KJ/mol compared to 48.1 KJ/mol for the fermentation of butyrate to acetate and H_2). For anaerobic bioremediation of CAHs, the control of TBOS hydrolysis rates would be important to achieve a high efficiency of utilization for dechlorination.

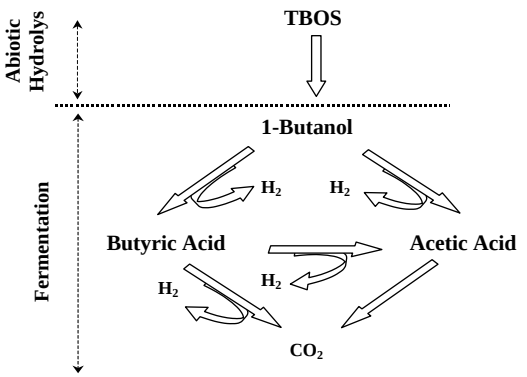

Fig. 5. Proposed anaerobic transformation pathway of TBOS. Adapted from Vancheeswaran [18]. Possible fermentation of butanol to butyrate and acetate: $2C_4H_9OH + 2HCO_3^- \rightarrow 2C_3H_7COO^- + CH_3COO^- + H^+ + 2H_2O$ [20], and butyrate to acetate: $C_3H_7COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$ [27].

3.5. Rates of TBOS hydrolysis

The abiotic hydrolysis rate of TBOS governed the rate of butanol production. Several factors for the abiotic hydrolysis of alkoxysilanes and other silicon polymers (i.e., pH, moisture content in soil, and exchangeable cations) have been considered by other researchers [15,18,21,22] in the abiotic hydrolysis of different types of alkoxysilanes. In this study, several factors related to TBOS hydrolysis to butanol were monitored and compared with experiment data obtained here and other data obtained with batch vials with Ottawa sand (Table 3).

At initial concentrations of 50 mg/l (156 μM), TBOS hydrolyzes at zero-order rates of 0.87 and 0.49 μM/day, in LLNL groundwater and Ottawa sand, respectively (Table 3(a) and (b)). The hydrolysis rate of TBOS in the bioaugmentation microcosm at an initial concentration of 90 mg/l (278 μM) (Fig. 3(a)) was 1.2 μM/day (Table 3(d)). This rate was slightly higher than the rate of 0.87 μM/day (Table 3(e)) obtained in the sterile control with Ottawa sand and the carbonate buffer (pH 7.2). Increasing TBOS concentrations resulted in more rapid abiotic hydrolysis rates (Table 3(d) and (e)) as compared with the LLNL groundwater or Ottawa sand and carbonate buffer (Table 3(a) and (b)). Hydrolysis of TBOS as an LNAPL phase could be controlled by direct hydrolysis on the surface of suspended TBOS droplets [15]. Accordingly, the increases of zero-order rates with the higher TBOS concentrations could result from the greater surface area of TBOS LNAPL. Thus, the concentration of TBOS as an LNAPL phase is likely one factor controlling the rates of hydrolysis. The highest rate of TBOS hydrolysis was achieved in the $HgCl_2$ poisoned microcosm. It is possible that Hg^{2+} ion helped catalyze the hydrolysis of TBOS. Liu et al. [23], for example, found Hg^{2+} enhanced the hydrolysis of a herbicidal additive (Irgarol 1051). The results of our studies indicate that the hydrolysis observed in the anaerobic microcosms was abiotic and not biologically mediated, since rates of TBOS utilization were not faster in the microcosms that were biologically active.

3.6. Electron mass balances

Electron mass balances were calculated for the three different microcosm studies by comparing the concentrations of TBOS, TKEBS, alcohols, acids, CAHs, and methane before and after dechlorination (Table 4). The data in Table 4 represents the averages of the duplicate microcosms. Excellent electron mass balances were achieved at the end of the incubations, ranging from

Table 3
Hydrolysis rates of TBOS based on the disappearance of TBOS

	Environmental conditions				Rate of hydrolysis (μM/day) based on the disappearance of TBOS
	Temp (°C)	Initial concentration μM (mg/l)	Solids	Water	
(a)	30	156 (50)	N/A	LLNL site 300	0.87[a]
(b)	20	156 (50)	Ottawa sand	Carbonate buffer	0.49[b]
(c)	30	156 (50)	N/A	LLNL site 300	1.27[c]
(d)	20	278 (90)	N/A	LLNL site 300	1.2[d]
(e)	20	278 (90)	Ottawa sand	Carbonate buffer	0.87[b]

[a] From Fig. 2(a).
[b] From our hydrolysis tests.
[c] From Fig. 1(a) ($HgCl_2$ poisoned).
[d] From Fig. 3(a).
All rates were obtained from a simple linear regression fit.

Table 4
Electron balances for the three different microcosms

	e^- equiv. per mole	e^- μ equivalents					
		LLNL		LLNL with the Evanite culture		Pt. Mugu	
		Day 0	Day 180	Day 0	Day 100	Day 511	Day 551
TKEBS	144[a]	3427	2304	0	0	0	0
TBOS	96[a]	3446	950	2006	989	142,080	134,400
Ethylbutanol	36[b]	0	0	0	0	0	0
Butanol	24[b]	0	115	0	0	0	0
Ethylbutyrate	32[b]	8467	8233	0	0	0	0
Butyrate	20[b]	356	602	0	0	720	2654
Acetate	8[c]	436	2037	149	690	17,600	23,893
c-DCE	2	0	514	0	0	0	0
VC	4	0	0	0	4	0	279
Ethylene	6	0	0	0	5	0	2
CH_4	8[c]	0	0	0	321	1076	3432
Total		16,132	14,755	2155	2009	161,476	164,660
Recovery (%)			91		93		102

[a] Based on the assumption that 1 mol of TBOS/TKEBS hydrolyzes to 4 mol of butanol/ethylbutanol.
[b] Based on the following equations:
Ethylbutanol: $C_6H_{13}OH + 11H_2O \rightarrow 6CO_2 + 36H^+ + 36e^-$
Butanol: $C_4H_9OH + 7H_2O \rightarrow 4CO_2 + 24H^+ + 24e^-$
Ethylbutyrate: $C_5H_{11}COO^- + 11H_2O \rightarrow HCO_3^- + 5CO_2 + 32H^+ + 32e^-$
Butyrate: $C_3H_7COO^- + 7H_2O \rightarrow HCO_3^- + 3CO_2 + 20H^+ + 20e^-$
[c] Sawyer et al. [24].

91% to 102%. The electron flow into dechlorination was low, ranging from 1% to 14%. These dechlorination estimates were based on the electron equivalents utilized in the dechlorination of TCE to the amounts of TBOS and TKEBS hydrolyzed. This is a conservative estimate, since the electron donor potential in the organic acids and alcohols formed was not considered. In our study, most of the electron flow went into the creation of organic acids, especially acetate, which ranged from 44% to 82%. Electron flow to methanogenesis increased from 0% in the LLNL microcosms that were not bioaugmented to 31% in the bioaugmented microcosms. Also, CAH concentrations were maintained at low levels in these microcosm tests, which likely lowered the efficiency of the electron transfer to dehalogenation and heightened that to acetogenesis.

4. Conclusions

TBOS abiotically and slowly hydrolyzes to 1-butanol, which ferments to butyrate and/or acetate, producing H_2 during the fermentation process. One mol of TBOS was shown to produce 4 mol of 1-butanol through abiotic hydrolysis. Butanol ferments to butyrate and/or acetate, consistent with the observations of Eichler and Schink [20]. Butyrate could then serve as a slowly fermenting substrate to drive reductive dechlorination. Butyrate has also been reported as a low-H_2-producing substrate favored by dechlorinators rather than methanogens [9].

Based on the results of microcosm study, TBOS was found to be a slow-release anaerobic substrate to support long-term dechlorination in LLNL microcosms, Pt. Mugu microcosms, and the LLNL microcosm bioaugmented with the Evanite culture. These results indicate that TBOS has promise as an effective anaerobic substrate for remediating a wide range of CAH concentrations at many contaminated sites. First, it may be possible to maintain a rate of TBOS abiotic hydrolysis that matches that required to achieve effective in situ bioremediation, if the initial TBOS amount introduced is carefully controlled according to the mass of CAH contaminants. This could provide a better environment for dechlorinators to outcompete the other hydrogenotrophs such as methanogens, sulfidogens, and acetogens. Second, high operational costs resulting from repeated or continuous injections of commonly used anaerobic substrates such as lactate, butyrate, propionate, and benzoate might be significantly reduced through the use of a slow-release substrate, such as TBOS. TBOS as an LNAPL with a low solubility in water might be injected into the subsurface to create a barrier that slowly releases butanol. The results of these microcosm studies show a single addition of TBOS will produce an electron donor at a slow rate to drive anaerobic transformations for extended periods of time. The commercial availability of TBOS at a low cost may limit its use. Analytical grade TBOS costs about $17 per pound, however the commercial cost should be lower. Since it is not a food or food additive, obtaining regulatory approval for its addition may prove more difficult than other slow-release substrates such as vegetable oil. TBOS might be effectively used to treat a DNAPL zone through a single addition. Anaerobic substrates have been widely used for in situ CAH bioremediation, including lactate, butyrate, propionate, benzoate, and hydrogen [4,5,9]), but these substrates need continuous injection. Slowly fermentating vegetable oil, for example, has been implemented at several CAH-contaminated sites [25,26]). As observed in the vegetable oil studies, chlorinated solvents could easily partition into TBOS injected near a DNAPL zone [14], thus reducing the aqueous concentrations of PCE or TCE, and potentially reducing toxicity and inhibition. Thus, these concentration reductions may actually enhance rates of reductive dehalogenation. This potential treatment of NAPL phases using TBOS needs to be studied in future work, as well as treatments under conditions that are more representative of in situ bioremediation.

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